Differential regulation of microRNA transcriptome in chicken lines resistant and susceptible to necrotic enteritis disease

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ABSTRACT Necrotic enteritis (NE) is a re-emerging disease as a result of increased restriction on the use of antibiotics in poultry. However, the molecular mechanisms underlying the pathogenesis of NE are unclear. Small RNA transcriptome analysis was performed using spleen and intestinal intraepithelial lymphocytes (IEL) from 2 inbred chicken lines selected for resistance or susceptibility to Marek's disease (MD) in an experimentally induced model of avian NE to investigate whether microRNA (miRNA) control the expression of genes associated with host response to pathogen challenge. Unique miRNA represented only 0.02 to 0.04% of the total number of sequences obtained, of which 544 were unambiguously identified. Hierarchical clustering revealed that most of miRNA in IEL were highly expressed in the MD-susceptible line 7.2 compared with MD-resistant line 6.3. Reduced *CXCL14* gene expression was correlated with differential expression of several unique miRNA in MD-resistant chickens, whereas *TGFβR2* gene expression was correlated with altered gga-miR-216 miRNA levels in MD-susceptible animals. In conclusion, miRNA profiling and deep sequencing of small RNA in experimental models of infectious diseases may be useful for further understanding of host-pathogen interactions, and for providing insights into genetic markers of disease resistance.

Key words: chicken, next-generation sequence, microRNA, necrotic enteritis, *Eimeria maxima*, *Clostridium perfringens*

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INTRODUCTION

One family of the small RNA transcriptome comprises microRNA (**miRNA**), noncoding RNA approximately 21 to 25 nucleotides (**nt**) long that are found in both animals (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001) and plants (Llave et al., 2002; Park et al., 2002). The miRNA are complementary to the 3′-untranslated regions of one or more mRNA, and their interaction with the 3′- untranslated region usually triggers gene silencing via translational repression or target degradation (Bartel, 2009; Eulalio et al., 2009). The human genome encodes over 2,000 miRNA that may target up to 60% of expressed genes (Bentwich et al., 2005; Lewis et al., 2005; Friedman et al., 2009). The patterns of miRNA-regulated gene expression are conserved between mammalian and avian species, and recent developments in high throughput sequencing technology have allowed the identification of tens of millions of short or small RNA. Because immune genes have been shown to have a higher propensity for miRNA target sites (Hicks et al., 2009), studies of miRNA functions have mainly focused on a variety of human diseases, including congenital disorders (Mencia et al., 2009), cancer (He et al., 2005), heart disease (Thum et al., 2007), and obesity (Skarn et al., 2012) . Therefore, the association between miRNA-associated regulation or dysregulation and chicken diseases may increase our general understanding of immune system regulation in avian species (Bacon et al., 2000).

Necrotic enteritis (**NE**) in chickens has historically been controlled using in-feed ionophoric anticoccidials, which also possess antibacterial activity against *Clostridium perfringens*, the etiologic agent of NE. Recently, NE has re-emerged as a significant problem for the poultry industry as a result of restrictions on the use of antibiotics, modern practices of high-density production conditions on re-used litter (Williams, 2005). In the United States, NE is among the most important infectious diseases in chickens (Smith and Helm, 2008; Lee et al., 2011a). The economic loss due to NE is estimated to cost the US poultry industry more than $2 billion annually, largely attributable to medical treatments and impaired growth performance. Co-infection

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with *Eimeria* parasites and feeding a high protein diet are 2 common risk factors that predisposes meat-type (broiler) chickens to NE (Park et al., 2008). Clinical illness is usually very short, and often the only sign of infection is a sudden increase in mortality within the flock.

Currently, there are 791 known chicken miRNA in miRBase 19.0 (released August 2012). This is significantly lower than the number of miRNA in the mouse (1,281) and human (2,042), but higher than that of other livestock, such as cows (755), pigs (306), and sheep (103). MicroRNA expression in the chicken has been characterized in several processes, such as embryo development (Darnell et al., 2006; Hicks et al., 2008; Rathjen et al., 2009), germ cell development (Lee et al., 2011b), immune organ function (Hicks et al., 2009), and disease (Yu et al., 2008; Tian et al., 2012; Wang et al., 2013).

To date, however, there are no research reports describing the small RNA transcriptome changes associated with avian NE. Using 2 disparate genetic models, chicken lines 6.3 and 7.2, we induced experimental NE with *Eimeria maxima* and *Clostridium perfringens*, and investigated whether the small RNA transcriptome influences the level of protein-coding mRNA during NE infection.

Our results suggest that the differential expression of several unique miRNA may be associated with genetically determined NE disease susceptibility in chickens and that further understanding of how miRNA are regulated during the onset of NE in chickens will be critically important for the future design and implementation of disease-resistant breeding programs in commercial poultry flocks.

MATERIALS AND METHODS

Experimental Birds and NE Disease Model

Two highly inbred White Leghorn chicken lines, line 6.3 and line 7.2 were kindly provided by the Avian Disease and Oncology Laboratory (East Lansing, MI) of USDA-Agricultural Research Service. Each line has been selected and maintained for decades after exposure to avian leukosis virus (**ALV**) and Marek's disease (**MD**) virus (**MDV**); line 6.3 is resistant and line 7.2 is sensitive to these pathogens. To experimentally induce NE, chickens were infected as previously reported (Park et al., 2008; Jang et al., 2012). Chickens were infected with *E. maxima* strain 41A (1.0×10^4 oocysts/bird) by oral gavage on d 14 posthatch followed by oral gavage with *C. perfringens* strain Del-1 (1.0×10^9 cfu/bird) on d 18. To promote the induction of NE, birds were fed an antibiotic-free certified organic starter diet containing 17% CP between d 1 and 18 followed by a standard grower diet containing 24% CP between d 18 and 20. All protocols were approved by the Beltsville Area Institutional Animal Care and Use Committee of USDA-Agricultural Research Service.

Total RNA Extraction

On d 20 posthatch, spleen and intestinal intraepithelial lymphocytes (**IEL**) were collected from 5 chickens of each group. Briefly, spleens were carefully homogenized with a mortar and pestle after freezing with liquid nitrogen. Intestines were cut longitudinally and washed 3 times with ice-cold Hanks' balanced salt solution containing 100 U/mL of penicillin and 100 mg/mL of streptomycin (Sigma, St. Louis, MO). The mucosal or inner layer was carefully removed using a cell scrapper (Nunc, Thermo Scientific Inc., Waltham, MA). Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) as described (Hong et al., 2012).

Small RNA Deep Sequencing

Small RNA in the desired size range (18~30 nt) were eluted form denaturing 15% polyacrylamide gels and ligated with 3′ and 5′ adaptors to make a small RNA cDNA library as previously described (Hafner et al., 2008). The reverse-transcription reaction was performed and cDNA was synthesized using 5′ adaptor forward and 3′ adaptor reverse primers. The PCR products were sequenced by Theragen Bio Institute (Suwon, Korea) using a HiSeq 2000 high throughput sequencer (Illumina Inc., San Diego, CA).

Analysis of Sequence Data

The 50-nt sequence tags from HiSeq sequencing first went through a data cleaning process to remove low quality tags, 5′ and 3′ primer contaminants, sequences without the insert tag, poly A tags, and tags shorter than 18 nt. Subsequently, standard bioinformatics analysis was carried out to align or annotate the remaining tags into the chicken genome by Rfam 11.0 (http://rfam.sanger.ac.uk) and the GenBank database. In particular, small RNA were mapped to each chromosome in the sense or anti-sense orientation by Short Oligonucleotide Alignment Program (**SOAP**) software (http://soap.genomics.org.cn/soap1). Analysis of differential miRNA expression, and prediction of novel miRNA using unannotated small RNA or associated mRNA gene targets were performed using miRBase 19.0 (http://www.mirbase.org), Mireap (http://sourceforge.net/projects/mireap), and TargetScanHuman (http://www.targetscan.org).

Hierarchical Cluster Analysis for Known miRNA

Hierarchical cluster analysis was performed for known miRNA with similar expression patterns (Zhang et al., 2009). Samples from the spleen and IEL of lines 6.3 and 7.2 were compared as control and treatment, respectively. In cluster analysis, miRNA that showed similar patterns of differential expression in different sample pairs were clustered together.

miRNA Target Gene Prediction and Analysis

Because a database for predicting chicken target genes is not available, miRNA potential target genes were identified based on TargetScanHuman (http://www.targetscan.org). Immune-related genes have been indicated as the main target genes of miRNA in this report.

Pathway and Network Analyses

For selected immune-related genes indicated in Table 5, the network and pathway information of differentially expressed genes were analyzed by Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems Inc., Redwood City, CA). Bio-functions were grouped into categories of disease and disorders, molecular and cellular functions, and physiological system development and function. Canonical pathway and network analysis were performed in a similar manner (Jimenez-Marin et al., 2009). The *P*-values were calculated using the right-tailed Fisher's exact test.

Target Gene Expression Analysis

Five μg of total RNA was treated with 1.0 unit of DNase I and 1.0 μL of 10× reaction buffer (Thermo Scientific), and incubated for 30 min at 37°C. Subsequently, 1.0 μL of 50 m*M* EDTA was added to inactivate DNase I, and the mixture was heated to 65°C for 10 min. The RNA was reverse transcribed using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's recommendations. Briefly, 5.0 μg of RNA was combined with 5× reaction mix, 2.0 μL of Maxima Enzyme Mix, and RNase-free water to give a total volume of 20 μL. The mixture was incubated at 25°C for 10 min, followed by 15 min at 50°C, and the reaction was terminated by heating at 85°C for 5 min. After cDNA synthesis, gene expression profiles were detected and quantified using an equivalent amount of cDNA. The cDNA (100 ng) was added into a reaction mix including 10 μL of 2× Power SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA), 0.5 μL of each primer, and RNase-free water to a total volume of 20 μL. Two-step real-time PCR was performed by an ABI 7500 system using the following standard cycling program: 50°C for 2 min; 95°C for 2 min; 40 cycles of 95°C for 15 s, 65°C for 30 s. Each analysis was performed in triplicate. Standard curves were generated using $\log_{10}$ diluted cDNA from pooled total RNA as described (Hong et al., 2012). Data from quantitative reverse-transcription (**qRT**) PCR were normalized relative to the expression of *GAPDH* using the Q-gene program (Muller et al., 2002). Oligonucleotide primers for target genes and GAPDH control were designed based on sequences available from public databases using Lasergene software (DNASTAR Inc., Madison, WI) and are indicated in Table 1.

Statistical Analysis

For quantitative real-time PCR, mean ± SE values for each group (n = 5) on pooled samples were calculated, and the differences between groups were analyzed using the Student's *t*-test and IBM SPSS software (SPSS 20.0 for Windows, Chicago, IL). Differences were considered significant at $P < 0.05$. The *P*-values for Pathway and Network analyses were calculated using the right-tailed Fisher's exact test.

RESULTS

Distribution of Sequence Count Numbers of Chicken Small RNA

By next-generation sequence (**NGS**) analysis of spleen and IEL from 2 chicken lines co-infected with *E. maxima* and *C. perfringens,* a total of 82 million and 89 million sequence reads for the MD-resistant line 6.3 and MD-sensitive line 7.2 were obtained (Table 2). Unique small RNA read numbers were categorized into 12 groups (Figure 1). Most unique small RNA (68.9%) were unannotated, whereas miRNA (known and novel) represented from 0.02 to 0.044% (Figure 1). The relative proportions of unique small RNA are unannotated and annotated as miRNA, snRNA, tRNA, rRNA, siRNA, and mRNA (repeat, unannotated, and polII-transcribed) for the spleen and intestinal IEL of lines 6.3 and 7.2. MicroRNA ranked fourth in abundance among total small RNA, ranging from 8.7 to 13.4% of the total (data not shown). Among the unique small RNA, the proportion of miRNA was relatively lower, ranging from 0.02 to 0.04% of the total. Using SOAP, 69.3 to 78.6% of the total small RNA were chromosome mapped, and 70.3 to 75.0% of unique small RNA were mapped to the chicken genome (Table 2). The number and relative proportions of total and unique small RNA sequence read numbers in the spleen and IEL of the 2 chicken lines are compared in Table 3. For the total small RNA, the fraction of sequence counts common to lines 6.3 and 7.2 was higher in the spleen (81.8%) compared with IEL (71.1%). Total small RNA read numbers that were specific for spleen or IEL were greater in line 7.2 versus line 6.3 (10.4 vs. 7.8% in spleen; 17.5 vs. 11.4% in IEL). Both of these trends were also seen with the unique small RNA.

miRNA Identification and Target Gene Prediction

MicroRNA in the spleen and intestinal IEL of the 2 chicken lines were identified using miRBase 19.0. Five hundred forty-four miRNA were identified, consisting of the number of miRNA plus the number of miRNA* on the opposite arm of the miRNA precursor (Table 4). These miRNA/miRNA* numbers were essentially identical when comparing between the 2 chickens lines,

Table 1. The oligonucleotide sequences are used for real-time PCR on microRNA target genes

Gene[1]	F or R[1]	Primer sequence (5′-3′)	Size (bp)	GenBank accession no.
GAPDH	F	TGC TGC CCA GAA CAT CAT CC	142	NM_204305
	R	ACG GCA GGT CAG GTC AAC AA		
SOCS3	F	GAC ACC AGC CTG CGC CTC AAG A	111	NM_204600
	R	GCC CGT CAC CGT GCT CCA GTA GA		
SOCS6	F	CAG ATA TCT TTG TGG ACC AGG CAG TGA A	115	NM_001127312
	R	GGT AGC AAA GGT GAA AGT GGA GGG ACA TC		
BCL6	F	CCT TCG CAC CTG GCT TAT TCA ACA	118	NM_001012930
	R	GGC ATC CTC ATC TCC CGC AAC TCA		
IRF2	F	GAT CTC CCC TGT CTC GTC CTA TGC	110	NM_205196
	R	TGC CTT CAA TGT TTT TCT TCT GCC AAT G		
TRAF3	F	CGT CTC GGC GCC ACT TAG GA	97	XM_421378
	R	GGG CAG CCA GAC GCA ATG TTC A		
TNFRSF21	F	TTG GGA AAA ACA TGG TGG TGG TGA AG	114	NM_001031103
	R	CAT CCG CCT GGG CAT CCG ACG AA		
ALCAM	F	TGA ACC GGA GGA TAG AAA TGA TGA CA	102	NM_205179
	R	GCA ACC AGG GCA ACC AGC AGA AG		
CXCL14	F	GCC TTG CTT CTG CTG GTC ATC	88	NM_204712
	R	ATC TTA TTT TCG GCC CTT TCC TT		
COL1A2	F	CTC AGC TTT GTG GAT ACG CGG ATT TTG	91	NM_001079714
	R	GCC CTG CAG ATG CCT CAC TCA CA		
NFKBIZ	F	CCA GGT CCT CCA GGC AAT CCA AAA G	95	NM_001006254
	R	AGT GCA GGG CTG TCA AAC CAT CGT AG		
TNFRSF11B	F	CAT CTC GGC CAA CCA AGT CTC ACC T	92	NM_001033641
	R	CGC TCG ATA TCT TCT TTT CCC ACT TTC TTG		
CALB1	F	GAT CTC GGC CGC CCA GTT CTT	86	NM_205513
	R	AGT TTT GTA GCT CCT TCC CAT CCA T		
TGFBR2	F	TGC AAT TGA TCC CCA TAA GCC AGA G	91	NM_205428
	R	AGC AAC AGA AAT CAC CAA TAA AGG GAC AAG		
ARHGEF6	F	ACT GCT GGG AAA TGT GGA GGA AAT C	149	NM_001006432
	R	ACG TCA GGT ACA GGG AGC GGA ACT		
HSP90B1	F	CTG GCT CTG GCA TGC ACG CTT CT	121	NM_204289
	R	CTT CAT CAT CAG TTC GGG ACC CTT CTC TAC		
SERPINF1	F	CGG CAG CAG ACA AGG GGA AGG ATT	88	NM_001257289
	R	TGA AGT AAG CAG CCC CAG CAA GGA G		

[1]*ALCAM* = activated leukocyte cell adhesion molecule; *ARHGEF6* = Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6; *BCL6* = B-cell CLL/lymphoma 6; *CALB1* = calbindin 1, 28 kDa; *COL1A2* = collagen, type I, alpha 2; *CXCL14* = chemokine (C-X-C motif) ligand 14; *GAPDH* = glyceraldehyde 3-phosphate dehydrogenase; *HSP90B1* = heat shock protein 90 kDa beta (Grp94), member 1; *IRF2* = interferon regulatory factor 2; *NFKBIZ* = nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta; *SERPINF1* = serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1; *SOCS3* = suppressor of cytokine signaling 3; *SOCS6* = suppressor of cytokine signaling 6; *TGFBR2* = transforming growth factor, beta receptor II (70/80 kDa); *TNFRSF11B* = tumor necrosis factor receptor superfamily, member 11b; *TNFRSF21* = tumor necrosis factor receptor superfamily, member 21; *TRAF3* = TNF receptor-associated factor 3.

[2]F= forward; R = reverse.

or comparing between the 2 tissue samples. However, a difference was noted when comparing the numbers of unique small RNA or total small RNA that were matched to miRNA precursors for the identified miRNA. Thus, the number of unique/total small RNA were greater in the spleen of line 7.2 chickens compared with the spleen of line 6.3, whereas the number of unique/total small RNA were greater in the IEL of line 6.3 chickens compared with the IEL of line 7.2. Next, the target genes of these identified miRNA were predicted using

Table 2. Mapping the small RNA tags to genome by short oligonucleotide alignment program (SOAP) to analyze their expression and distribution on the genome

	Line 6.3		Line 7.2	
Item	Spleen	IEL[1]	Spleen	IEL
Total sRNA				
Total sRNA	40,864,613	44,150,080	41,665,229	45,600,491
Percent	100	100	100	100
Mapping to genome	29,519,926	30,643,758	28,868,163	35,818,935
Percent	72.2	69.4	69.3	78.6
Unique sRNA				
Unique sRNA	6,616,085	9,133,836	8,232,973	10,970,078
Percent	100	100	100	100
Mapping to genome	4,652,262	6,855,088	5,978,863	8,169,540
Percent	70.3	75.0	72.6	74.5

[1]IEL = intraepithelial lymphocytes.

Table 3. The number and portion of common and specific small RNA sequences between lines 6.3 and 7.2 in spleen and intestinal intraepithelial lymphocytes (IEL) after necrotic enteritis (NE) induction with *Eimeria maxima* and *Clostridium perfringens*

Class	Unique sRNA	Percent	Total sRNA	Percent
Spleen				
Total sRNA	13,411,015	100.00	82,529,842	100.00
Line 7.2 and line 6.3	1,438,043	10.72	67,499,967	81.79
Line 7.2 spleen specific	6,794,930	50.67	8,579,671	10.40
Line 6.3 spleen specific	5,178,042	38.61	6,450,204	7.82
IEL				
Total sRNA	18,641,423	100.00	89,750,571	100.00
Line 7.2 and line 6.3	1,462,491	7.85	63,819,062	71.11
Line 7.2 IEL specific	9,507,587	51.00	15,725,394	17.52
Line 6.3 IEL specific	7,671,345	41.15	10,206,115	11.37

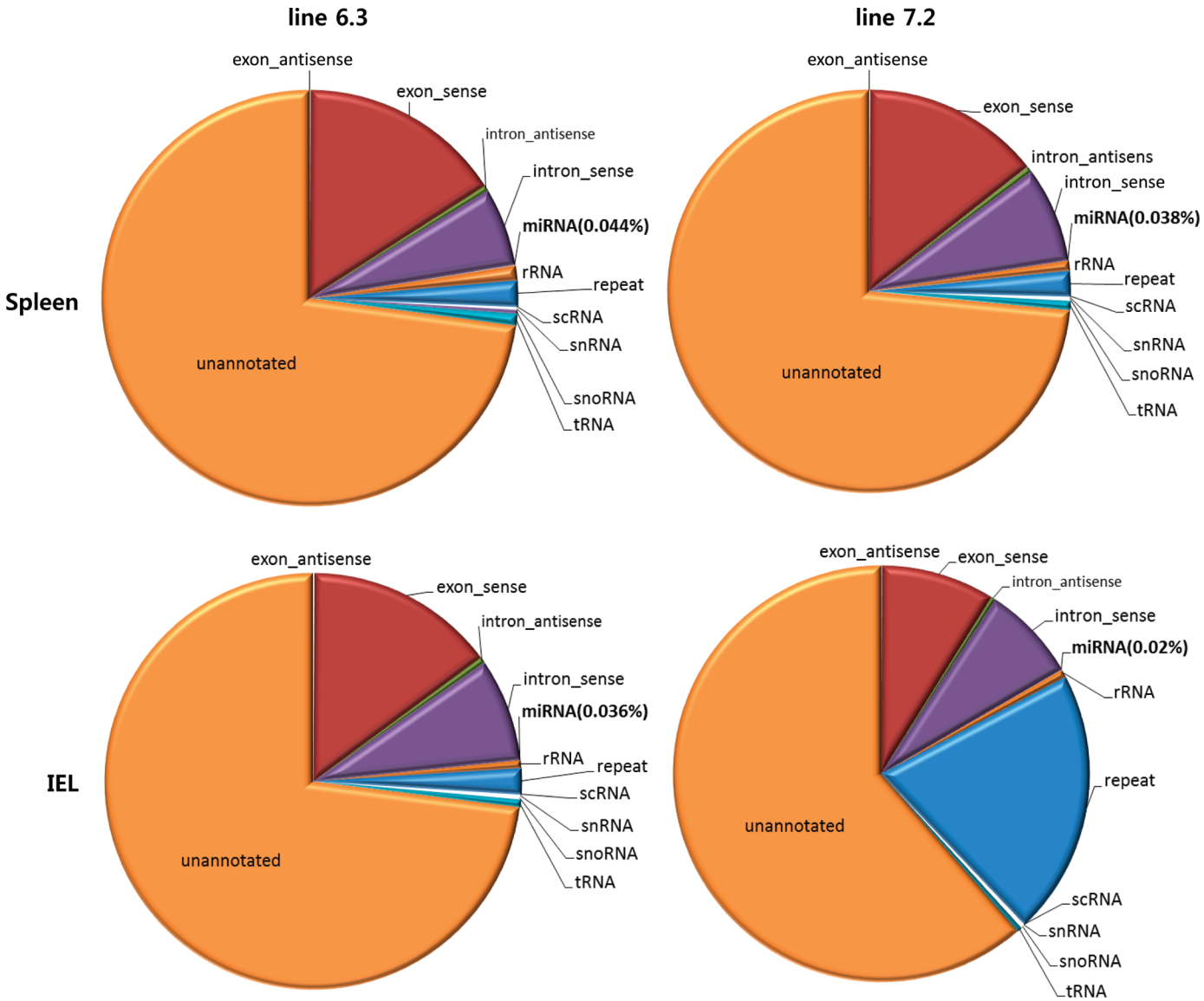

Figure 1. Pie chart for annotation of unique small RNA distribution among the different categories. The numbers of unique small RNA sequence reads for both genetically disparate lines after necrotic enteritis induction are shown in the spleen and intestinal intraepithelial lymphocytes (IEL). miRNA = microRNA; scRNA = small cytoplasmic RNA; rRNA = ribosomal RNA; snRNA = small nuclear RNA; snoRNA = small nucleolar RNA; tRNA = transfer RNA. Color version available in the online PDF.

Table 4. Summary of known microRNA (miRNA) in each sample

Item	miRNA	miRNA[1]	miRNA precursors	Unique small RNA matched to miRNA precursors	Total small RNA matched to miRNA precursors
Known miRNA in miRBase	467	77	499	—	—
Spleen line 6.3	249	28	277	3,154	3,570,481
Spleen line 7.2	250	27	278	3,358	5,254,632
IEL[2] line 6.3	261	30	294	3,520	5,931,769
IEL line 7.2	226	21	255	2,980	4,310,127

[1]Opposite arm of the precursor.

[2]IEL = intraepithelial lymphocytes.

Table 5. Comparison of the number of microRNA (miRNA) reads and their corresponding target genes between the spleen and intestinal intraepithelial lymphocytes (IEL) of lines 6.3 and 7.2

	Spleen		Intestinal IEL		
miRNA	Line 6.3	Line 7.2	Line 6.3	Line 7.2	Immune-related gene[1]
gga-let-7a	547,582	810,866	870,984	72,209	*COL1A2*
gga-let-7b	231,715	319,567	277,864	24,934	*COL1A2*
gga-let-7c	111,701	95,084	94,845	7,801	*COL1A2*
gga-let-7d	205	346	338	52	*COL1A2*
gga-let-7f	307,063	384,815	493,884	53,416	*COL1A2,*
gga-let-7i	5,331	7,789	7,579	1,392	*COL1A2*
gga-let-7j	545,962	808,199	867,621	72,158	*COL1A2*
gga-let-7k	199,177	224,746	317,690	13,762	*COL1A2*
gga-miR-101	68,030	106,686	116,933	12,539	*GJA1*, *ALCAM*, *APP*
gga-miR-106	2,445	3,718	4,559	576	*TGFBR2*, *VEGFA*, *TNFRSF21*, *CXCL14*, *SOCS6*
gga-miR-10a	212,995	277,674	286,941	2,493	*BCL6*
gga-miR-10b	549	796	821	209	*BCL6*
gga-miR-128	867	640	1,051	80	*SOCS6*
gga-miR-130a	346	348	291	64	*CALB1*
gga-miR-130b	3,085	2,627	3,152	389	*SOCS6*, *CALB1*
gga-miR-130c	1,200	1,241	1,522	104	*SOCS6*, *CALB1*
gga-miR-1329	621	1,325	1,443	440	*TCF12*, *ALCAM*, *NFKBIZ*
gga-miR-138	195	299	291	42	*TCF12*, *SOCS6*
gga-miR-142–3p	28,104	57,789	75,764	1,918	*TAB2*, *APP*
gga-miR-144	37	132	67	10	*ALCAM*
gga-miR-148a	216,402	183,601	278,762	108,423	*ALCAM*
gga-miR-155	4,013	7,522	7,899	150	*TRAF3*
gga-miR-1563	172	278	234	89	*SOCS6*
gga-miR-15a	242	345	279	36	*VEGFA*, *TAB3*, *BCL2*, *SOCS6*, *TRAF3*
gga-miR-15b	350	400	337	61	*SOCS6*, *TRAF3*, *BCL2*, *VEGFA*, *TAB3*
gga-miR-15c	291	449	412	87	*VEGFA*, *TAB3*, *BCL2*, *SOCS6*, *TRAF3*
gga-miR-16	2,885	5,172	4,384	727	*VEGFA*, *TAB3*, *BCL2*, *SOCS6*
gga-miR-1674	92	293	271	16	*TGFBR2*
gga-miR-16c	12,813	23,842	27,819	2,492	*VEGFA*, *BCL2*
gga-miR-17–5p	6,183	7,150	8,661	903	*TNFRSF21*, *CXCL14*, *SOCS6*, *VEGFA*
gga-miR-1781	301	774	812	87	*TRAF3*
gga-miR-181a	26,896	30,456	34,385	1,347	*CXCL14*, *BCL2*, *TAB3*
gga-miR-181b	5,570	8,495	9,113	506	*CXCL14*, *BCL2*, *TNFRSF11B*, *TAB3,*
gga-miR-183	213	1,480	1,357	83	*TCF12,SOCS6*
gga-miR-18a	339	556	683	77	*IRF2*, *ALCAM*
gga-miR-18b	234	411	557	68	*IRF2*, *ALCAM*
gga-miR-196	16	48	53	3,004	*COL1A2*, *IGF2BP3*
gga-miR-199	1,580	1,242	1,449	55	*TRAF3*, *VEGFA*
gga-miR-19a	553	680	903	101	*SOCS3*, *SOCS1*, *SOCS6*, *TAB3*
gga-miR-19b	923	940	1,343	150	*SOCS1*, *SOCS3*, *SOCS6*, *TAB3*
gga-miR-20a	7,563	12,303	15,088	1,589	*TNFRSF21,VEGFA*, *SOCS6*, *CXCL14*
gga-miR-20b	6,276	13,244	15,915	2,153	*SOCS6*, *CXCL14*, *TNFRSF21*, *VEGFA*
gga-miR-2131	68	129	72	36	*TCF12*, *IRF2*
gga-miR-215	1,794	2,170	5,848	335,4759	*ALCAM*
gga-miR-216	17	3	8	9,307	*TGFBR2*
gga-miR-216b	6	10	12	3,179	*SOCS6*
gga-miR-221	692	915	1,010	433	*TCF12*, *IRF2*, *BCL11B*, *SOCS1*
gga-miR-222	181	225	289	149	*TCF12*, *IRF2*, *SOCS1*
gga-miR-223	1,308	1,395	1,813	22	*HSP90B1*
gga-miR-23b	156	125	142	21	*SOCS6*, *TGFBR2*, *IRF2*
gga-miR-26a	39,709	46,919	48,669	6,129	*COL1A2*
gga-miR-27b	2,503	3,565	3,963	425	*SOCS6*, *TAB3*
gga-miR-2964	2,655	4,200	4,517	544	*TCF12*, *APP*

Continued

Table 5 (Continued). Comparison of the number of microRNA (miRNA) reads and their corresponding target genes between the spleen and intestinal intraepithelial lymphocytes (IEL) of lines 6.3 and 7.2

	Spleen		Intestinal IEL		
miRNA	Line 6.3	Line 7.2	Line 6.3	Line 7.2	Immune-related gene[1]
gga-miR-29b	138	159	177	137	*COL1A2*, *VEGFA*
gga-miR-29c	219	135	186	110	*COL1A2*
gga-miR-301b-3p	191	386	278	29	*SOCS6*, *CALB1*
gga-miR-30a-5p	118,223	128,741	140,203	11,033	*GJA1*, *ARHGEF6*, *BCL6*, *SOCS1*, *SOCS3*, *SOCS6*, *BCL2*
gga-miR-30b	427	258	369	41	*SOCS1*, *SOCS3*, *SOCS6*, *GJA1*, *BCL6*
gga-miR-30c	4,064	3,386	4,264	833	*ARHGEF6*, *SOCS1*, *SOCS3*, *GJA1*, *SOCS6*, *BCL6*
gga-miR-30d	119,635	180,939	174,940	31,394	*BCL6*, *SOCS1*, *SOCS6*, *SOCS3*, *BCL2*
gga-miR-30e	73,851	127,919	140,502	20,709	*ARHGEF6, GJA1*, *BCL6*, *SOCS1*, *SOCS6*, *SOCS3*
gga-miR-32	957	1,440	1,218	286	*SERPINF1*, *TRAF3*, *COL1A2*
gga-miR-33	153	273	228	125	*TRAF3*
gga-miR-34c	934	186	206	1	*TCF12*, *BCL2*
gga-miR-429	35	21	48	4,000	*VEGFA*, *TNFRSF11B*, *BCL2*
gga-miR-454	248	196	224	41	*SOCS6*, *CALB1*
gga-miR-455–5p	143	116	124	4	*CALB1*, *SOCS3*
gga-miR-7	1,105	2,054	2,562	1,178	*TAB2*, *COL1A2*
gga-miR-9	1,138	4,983	5,565	323	*BCL6*, *ALCAM*, *TRAF3*
gga-miR-92	15,234	21,750	23,337	5,914	*SERPINF1*, *TRAF3*, *COL1A2*

[1]*ALCAM* = activated leukocyte cell adhesion molecule; *APP* = amyloid beta (A4) precursor protein; *ARHGEF6* = Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6; *BCL2* = B-cell CLL/lymphoma 2; *BCL6* = B-cell CLL/lymphoma 6; *CALB1* = calbindin 1, 28 kDa; *COL1A2* = collagen, type I, alpha 2; *CXCL14* = chemokine (C-X-C motif) ligand 14; *GJA1* = gap junction protein, alpha 1, 43 kDa; *HSP90B1* = heat shock protein 90 kDa beta (Grp94), member 1; *IRF2* = interferon regulatory factor 2; *NFKBIZ* = nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta; *SERPINF1* = serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1; *SOCS1* = suppressor of cytokine signaling 1; *SOCS3* = suppressor of cytokine signaling 3; *SOCS6* = suppressor of cytokine signaling 6; *TAB2* = TGF-beta activated kinase 1/MAP3K7 binding protein 2; *TAB3* = TGF-beta activated kinase 1/MAP3K7 binding protein 3; *TCF12* = transcription factor 12; *TGFBR2* = transforming growth factor, beta receptor II (70/80 kDa); *TNFRSF11B* = tumor necrosis factor receptor superfamily, member 11b; *TNFRSF21* = tumor necrosis factor receptor superfamily, member 21; *TRAF3* = TNF receptor-associated factor 3; *VEGFA* = vascular endothelial growth factor A.

TargetScanHuman. Table 5 lists these genes and summarizes the differential expression patterns based upon read numbers of the corresponding miRNA. Some miRNA were more highly expressed in the spleen and IEL of MD-susceptible line 7.2 chickens compared with line 6.3, whereas others were more abundant in the MD-resistant line 6.3 versus line 7.2. *CXCL14* [chemokine (C-X-C motif) ligand 14] is of particular interest because of its known role in inflammation, and several miRNA associated with *CXCL14*, including gga-miR-20a, gga-miR-20b, gga-miR-106, gga-miR-17–5p, gga-miR-181a, and gga-miR-181b, were differentially expressed in line 6.3 versus line 7.2 chickens. However, differences were noted in the *CXCL14*-associated miRNA expression patterns when comparing the 2 tissues. For example, gga-miR-20a and gga-miR-20b expression levels in the spleen of line 7.2 were greater compared with the spleen of line 6.3, whereas both of these miRNA were greater in the IEL of line 6.3 vs. IEL of line 7.2. In contrast, no differences in gga-miR-106 levels were seen between the spleen of lines 6.3 and 7.2, whereas read numbers of this miRNA were greater in IEL of line 6.3 versus line 7.2. In another example, the expression of gga-miR-216, which was associated with *TGFβR2* (transforming growth factor, β receptor II), was higher in IEL of line 7.2 compared with IEL of line 6.3. Finally, Table 6 lists the total number of unique miRNA and their target gene numbers predicted by Mireap software. Both spleen and IEL of line 7.2 chickens contained higher numbers of unique miRNA and predicted target genes compared with line 6.3.

Hierarchical Cluster Analysis for Known miRNA

Hierarchical cluster analysis of 139 and 149 miRNA from the spleen and IEL, respectively, was performed based on their read numbers and a critical *P*-value of $P < 0.01$ was used to denote statistical significance. The expression of most spleen miRNA from the 2 lines was variable, and no distinct differences were detected between them (Figure 2). In IEL, however, most of miRNA were highly expressed in the MD-susceptible 7.2 line compared with the 6.3 line (green). However, a smaller subset of these miRNA (including gga-miR-217) showed the opposite pattern of expression where they showed higher expression in the resistant 6.3 line compared with the 7.2 line (red; enlarged panel, Figure 2).

Pathway and Network Analysis

Pathway and network analysis was performed for selected miRNA-associated, immune-related target genes that are indicated in Tables 1 and 5. Bio-functions were grouped into the following categories: disease and disorders, molecular and cellular functions, and physiological system development and functions. Biological function analysis, canonical pathway analysis, and network analysis were performed. For diseases and disorders, most target genes were associated with organismal injury and abnormalities, inflammatory responses, immunological disease, or cancer (Supplemental Table S1; http://dx.doi.org/10.3382/ps.2013-03666). For mo-

Table 6. The number of novel microRNA (miRNA) candidates whose target can be predicted and the number of predicted targets using those miRNA

Sample name		Number of unique miRNA	Target number
Spleen	Line 6.3	301	278,916
	Line 7.2	547	455,238
IEL[1]	Line 6.3	508	407,522
	Line 7.2	894	716,296

[1]IEL = intraepithelial lymphocytes.

lecular and cellular functions, target genes were significantly associated with cellular development, cellular growth and proliferation, or cellular movement. For physiological system development and functions, target genes were related to hematological system development and function, tissue morphology, or humoral immune responses. Canonical pathway analysis revealed that the selected immune-associated genes were most significantly associated with type I diabetes mellitus signaling, hepatic fibrosis/hepatic stellate cell activation, T helper cell differentiation, role of JAK2 in hormone-like cytokine signaling, and IL-6 signaling (Supplemental Table S2; http://dx.doi.org/10.3382/ps.2013-03666). Network analysis of these genes identified embryonic development, hematological development, cellular development, and hematopoiesis as top functions (Supplemental Table S3; http://dx.doi.org/10.3382/ps.2013-03666).

Quantitative RT-PCR of Differentially Expressed miRNA Target Genes

The transcript expression levels of 16 immune-related genes that were associated with differentially expressed miRNA in the MD-resistant and MD-sensitive chicken lines were measured by qRT-PCR and normalized to mRNA for GAPDH. The levels of transcripts encoding

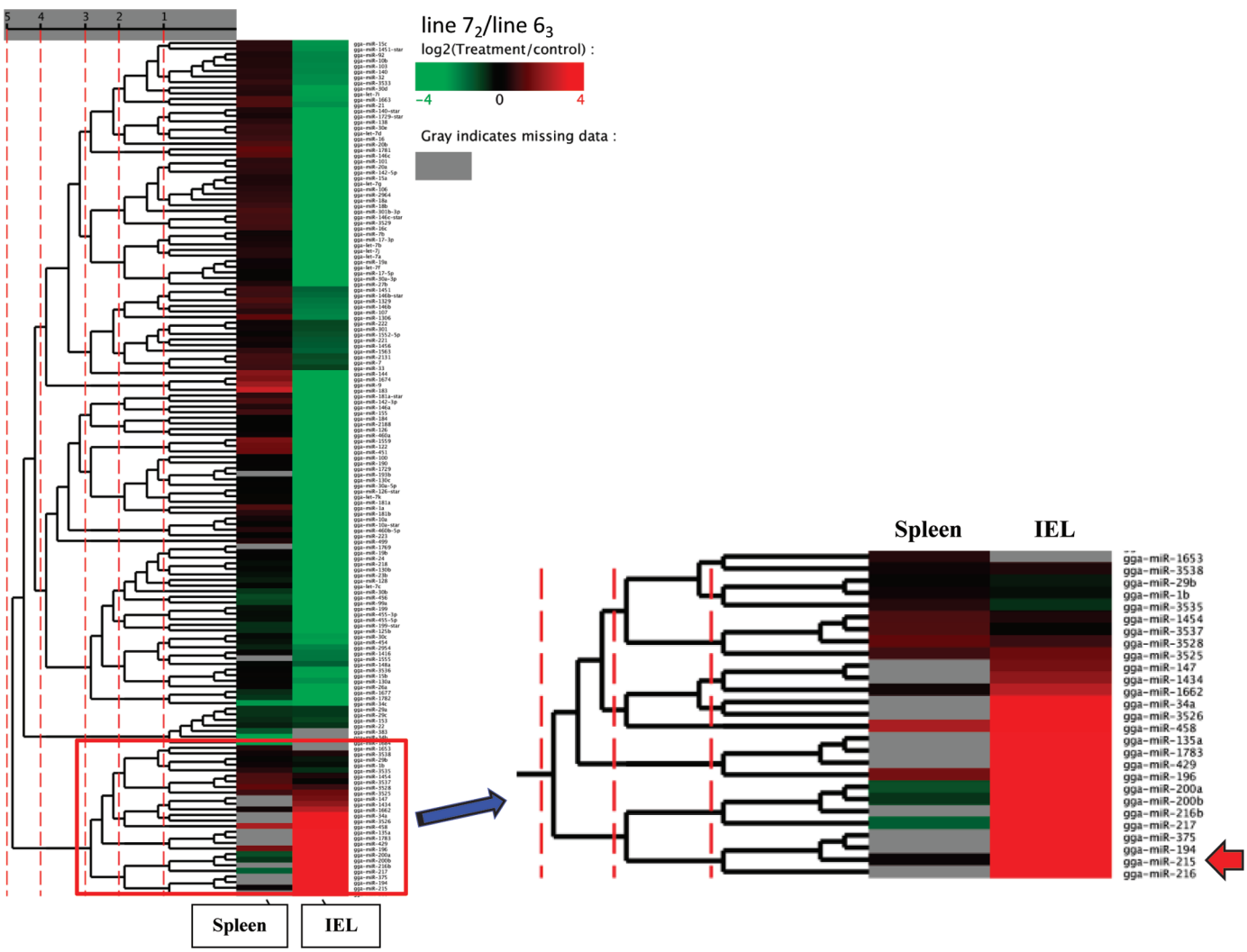

Figure 2. Hierarchical clustering of microRNA (miRNA) with similar expression patterns. Red indicates significantly higher miRNA expression in line 7.2 (Marek's disease susceptible). Green indicates significantly higher miRNA expression in line 6.3 (Marek's disease resistant). Gray indicates that the miRNA was not detected in at least 1 sample. Each row shows 1 miRNA and each column shows 1 sample pair (spleen and intestinal intraepithelial lymphocytes, IEL). The red arrow in the enlarged figure indicates gga-miR-217. The cutoff for significance was $P < 0.01$. Color version available in the online PDF.

SOCS6, *BCL6*, *IRF2*, *TRAF3*, *TNFRSF21*, *COL1A2*, *NFKBIZ*, *TNFSF11β*, *TGFβR2*, *ARHGEF6*, *HSP90β1*, and *SERPINF1* genes were significantly greater in the spleen, intestinal IEL, or both, of line 6.3 chickens compared with line 7.2 (Figure 3). On the other hand, transcripts for *SOCS3*, *ALCAM*, *CXCL14*, and *CALAB1* genes were significantly greater in the spleen, IEL, or both, of line 7.2 compared with line 6.3 chickens. Given that the expression of many target genes can be regulated by several different miRNA, we also analyzed the number of miRNA reads with the differential expression levels of the denoted mRNA. Quantification of the miRNA read counts revealed that the expression patterns of some miRNA were correlated with mRNA expression levels. For example, gga-miR-30b, gga-miR-30c, and gga-miR-455–5p read numbers inversely correlated with *SOCS3* transcript levels in the spleen and IEL of both lines. A similar inverse correlation was seen between *COL1A2* transcript levels and gga-miR-196 and the gga-let-7 series of miRNA read numbers, as well as between *CALB1* transcript levels and gga-miR-130b read numbers, in both spleen and IEL. The *CXCL14* mRNA levels were inversely correlated with read numbers for the gga-miR-181a and gga-miR-181b miRNA in the spleen of the 2 lines, but not in the IEL. Inverse associations also were observed between (a) *TNFSF11β* transcript levels and gga-miR-429 read numbers, (b) *HSP90β1* transcript levels and gga-miR-223 read numbers, (c) *NFKBIZ* transcript levels and gga-miR-1329 read numbers, (d) *TGFβR2* transcript levels and gga-miR-106 read numbers, (e) *ARHGEF* transcript levels and gga-miR-1674 read numbers, and (f) *SERPINF1* transcript levels and gga-miR-30e and gga-miR-32 read numbers.

DISCUSSION

Recent technological advances in small RNA NGS have facilitated our understanding of their profiles in the chicken and have led to the discovery of novel small RNA, including miRNA. Prior chicken miRNA deep sequencing analyses have been published in the context of embryo development (Darnell et al., 2006; Bannister et al., 2009; Hicks et al., 2010; Li et al., 2012) and cell growth and proliferation (Burnside et al., 2008). To our knowledge, this is the first report of small RNA high-throughput sequence analysis in an experimentally induced model of avian NE using 2 highly inbred chicken lines with well-characterized disease characteristics (Timbermont et al., 2009; Jang et al., 2012).

MicroRNA account for approximately 1.0 to 1.5% of all cellular transcripts and target at least one-third of all mRNA (Lewis et al., 2005; Bannister et al., 2009; Friedman et al., 2009). However, our data indicate that unique chicken miRNA account for 0.02 to 0.04% of the total deep sequence reads and approximately 8.7 to 13.4% of total small RNA, lower than reported in previous studies in humans (Morin et al., 2008). This discrepancy might be caused by different tissue functions , different responses to pathogens, or both, between mammals and birds. Further, because the line 7.2-specific small RNA read number is larger than line 6.3 in both spleen and IEL (Table 3), we suggest that small RNA (including miRNA) are expressed to inhibit translation of the target mRNA in the MD-sensitive line 7.2 versus line 6.3 chickens. These patterns were also found for novel miRNA candidates in the spleen and IEL of both lines (Table 5).

Hierarchical clustering of the identified miRNA provided an indication that these 2 inbred lines have different immune responses to the NE-associated pathogens, despite the fact that both were generated by inbreeding to select for resistance or susceptibility to MD (Bacon et al., 2000). In addition, the majority of miRNA expressed in the IEL of the MD-susceptible line 7.2 were overrepresented compared with line 6.3 (Figure 2). These results suggest that the expression of immune-related gene products that may be commonly regulated in response to these divergent pathogens (ALV/MDV vs. *E. maxima*/*C. perfringens*) is suppressed at the posttranscriptional level by miRNA in line 7.2 compared with line 6.3. Chicken microarray analysis has revealed that some miRNA, such as gga-miR-15b, are reduced in chickens susceptible to MDV (Tian et al., 2012). This result is consistent with our IEL data in NE-afflicted chickens (337 reads in line 6.3 vs. 61 reads in line 7.2; Table 5).

Among the target genes analyzed, the patterns of *CXCL14* and *TGFβR2* gene expression are particularly interesting because of their known roles in inflammatory responses. The *CXCL14* mediates its effects via selective activity in monocytes, dendritic cells, and natural killer cells (Kurth et al., 2001; Shurin et al., 2005; Starnes et al., 2006). Read numbers for some miRNA associated with the *CXCL14* gene were differentially regulated in lines 6.3 versus 7.2, and these changes were correlated with reduced *CXCL14* mRNA levels in line 6.3 in both the spleen and IEL (Figure 3). It is also evident, however, that the expression of some genes did not correlate with miRNA read numbers (Table 5, Figure 3). In mammals, imperfect matching between miRNA and the seed sequences of the target mRNA has been reported (Bartel, 2009), and some of the target genes predicted using the TargetScanHuman program may require independent confirmation (Garcia et al., 2011).

We observed that several novel candidate miRNA in the spleen and IEL of line 7.2 were more abundant compared with line 6.3. The numbers of total and unique small RNA in the spleen and IEL of line 7.2 were also highly expressed (Tables 2 and 6). This suggests that the differences in the expression of small RNA between the 2 chicken lines may be causally associated with their genetically disparate backgrounds, different responses to pathogens, or both. Additional studies will be required to verify the function of the miRNA identified in this report in experimentally induced avian NE. Future knowledge of the miRNA expression patterns in inbred

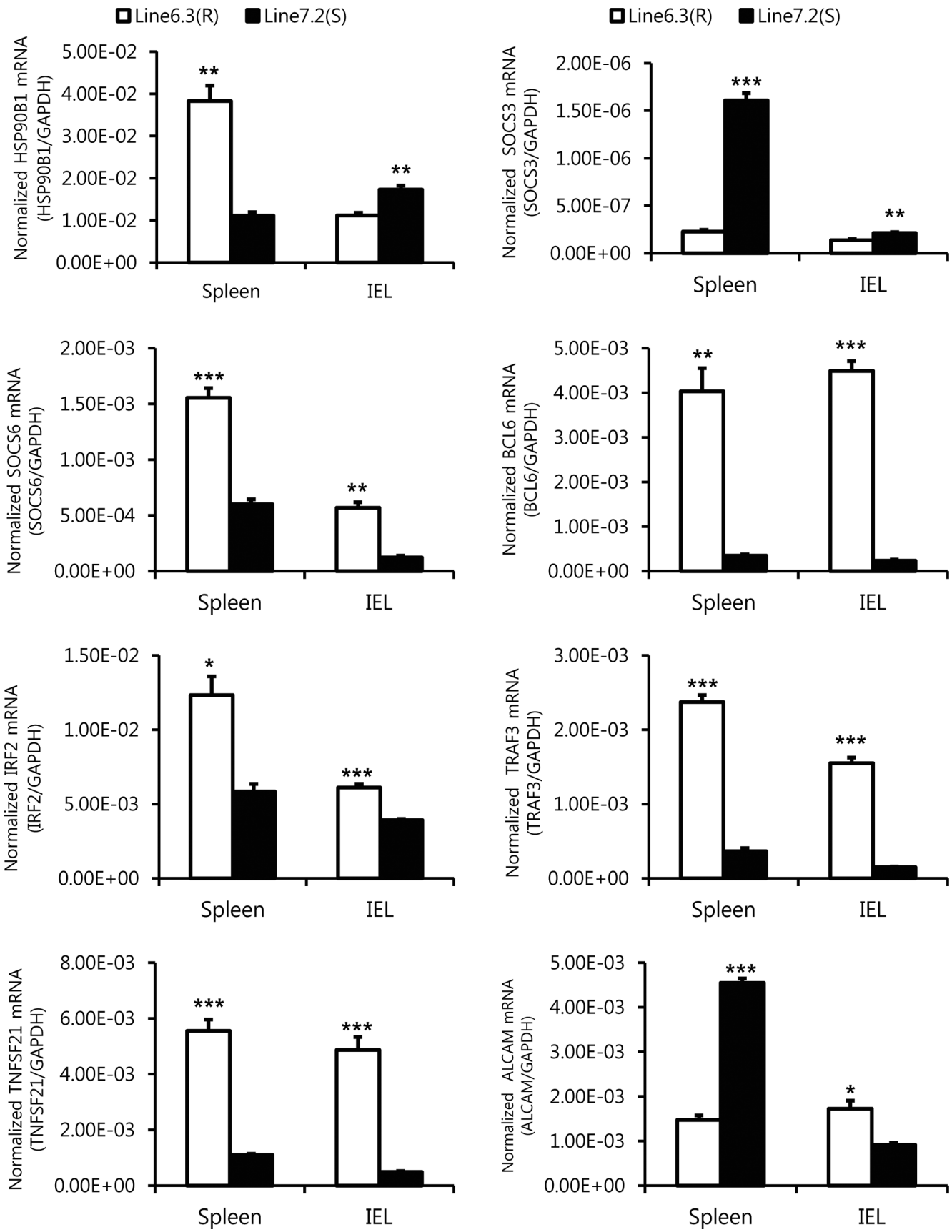

Figure 3. Transcript levels in the spleen and intestinal intraepithelial lymphocytes (IEL) of selected immune-related genes based on differential microRNA (miRNA) expression profiles. Transcript levels were determined by quantitative reverse-transcription PCR. Data are expressed as mRNA levels normalized to *GAPDH* mRNA levels from triplicate determinations with pooled samples from 5 chickens. *ALCAM* = activated leukocyte cell adhesion molecule; *BCL6* = B-cell CLL/lymphoma 6; *GAPDH* = glyceraldehyde 3-phosphate dehydrogenase; *IRF2* = interferon regulatory factor 2; *SOCS3* = suppressor of cytokine signaling 3; *SOCS6* = suppressor of cytokine signaling 6; *TNFRSF21* = tumor necrosis factor receptor superfamily, member 21; *TRAF3* = TNF receptor-associated factor 3. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

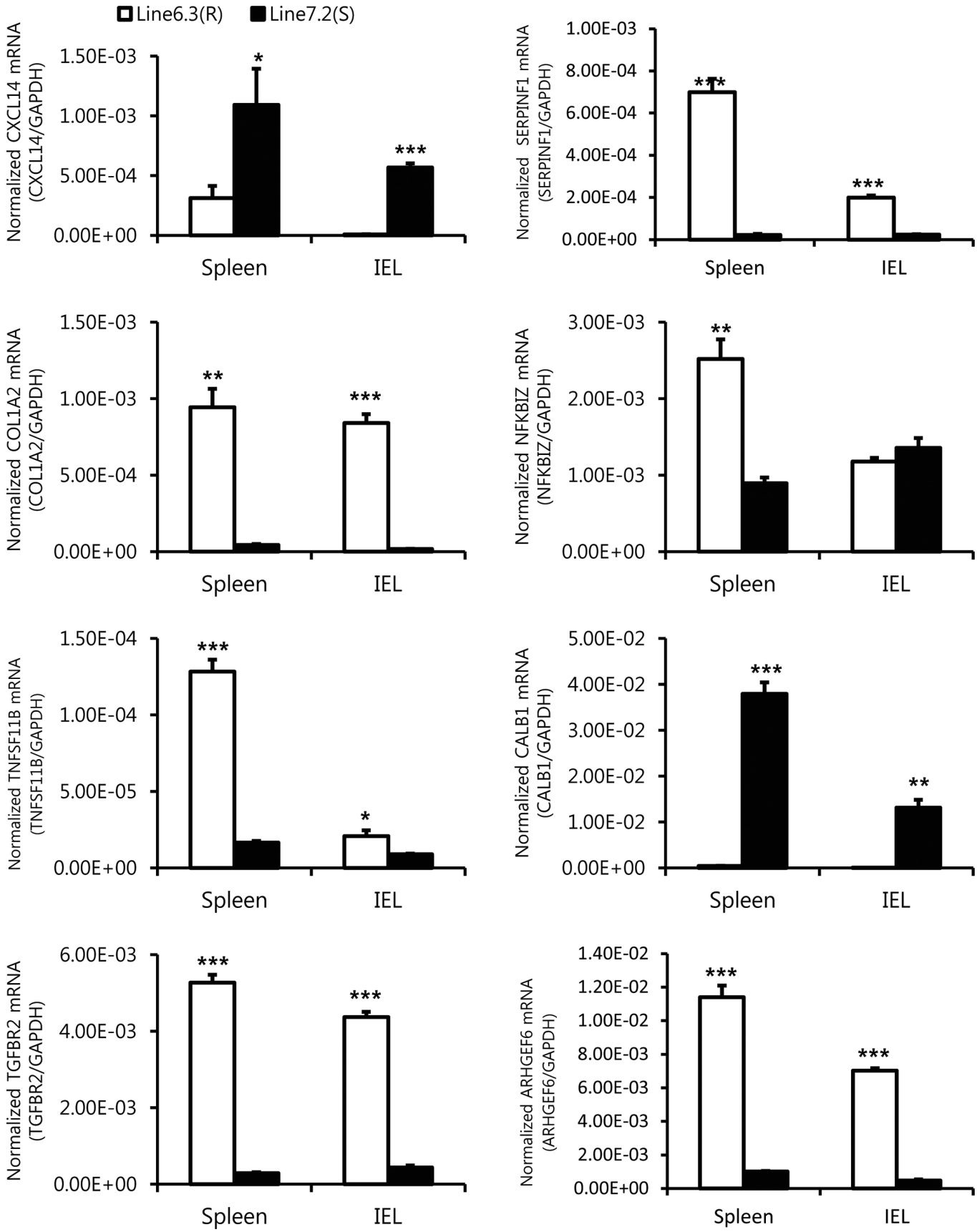

Figure 3 (Continued). Transcript levels in the spleen and intestinal intraepithelial lymphocytes (IEL) of selected immune-related genes based on differential microRNA (miRNA) expression profiles. Transcript levels were determined by quantitative reverse-transcription PCR. Data are expressed as mRNA levels normalized to *GAPDH* mRNA levels from triplicate determinations with pooled samples from 5 chickens. *ALCAM* = activated leukocyte cell adhesion molecule; *BCL6* = B-cell CLL/lymphoma 6; *GAPDH* = glyceraldehyde 3-phosphate dehydrogenase; *IRF2* = interferon regulatory factor 2; *SOCS3* = suppressor of cytokine signaling 3; *SOCS6* = suppressor of cytokine signaling 6; *TNFRSF21* = tumor necrosis factor receptor superfamily, member 21; *TRAF3* = TNF receptor-associated factor 3. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

chicken lines with different disease phenotypes might be useful for understanding how target genes are regulated by miRNA. These studies may also provide clues as to the identity of disease resistance genetic markers that could be incorporated into selective breeding programs used by commercial companies.

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